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(54) A PROCESS FOR THE PREPARATION OF α-1,6-GLUCOSIDASES

(71) We, HAYASHIBARA COMPANY, a Body Corporate organised and existing under the laws of Japan, of 2—3, 1-chome, Shimoishii, Okayama-shi, Okayama, Japan, do hereby declare the invention for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

The present invention relates to a process for the preparation of α-1,6-glucosidases which are similar in properties to isoamylase and pullulanase, and are capable of hydrolysing the α-1,6-glucosidic linkages of starch. Among the enzymes capable of hydrolysing the α-1,6-glucosidic linkages of polysaccharides, isoamylase obtained from yeast and potato, and pullulanase produced from *Aerobacter aerogenes* ATCC 8724 were previously known. Subsequently it was found that enzymes with capacities similar to isoamylases could be produced by cultivating strains of *Escherichia intermedia, Pseudomonas amyloderamosa, Actinomycetes,* and some of those of the genera *Lactobacillus, Micrococcus* and others.

These enzymes are capable of hydrolyzing the α-1,6-glucosidic linkages of polysaccharides. Although their actions on glycogen, dextrins and pullulan are somewhat individually different, their capacities of hydrolysing the α-1,6-glucosidic linkages of starch make the enzymes of great importance from the view of the starch hydrolysing industry.

We have now found pullulanase activities present in cultures of genera of *Corynebacterium, Aeromonas, Flavobacterium, Acetobacter, Vibrio, Actinoplanes, and Streptosporangium.* Further studies on the characteristics of the enzymes and their abilities to hydrolyse starch has resulted in the finding that the enzymes are applicable for industrial use.

According to the present invention there is provided a process for the preparation of α-1,6-glucosidases comprising cultivating *Corynebacterium sepedonicum* IFO 3306, *Aeromonas hydrophila* IFO 3820, *Flavobacterium esteroaromaticum* IFO 3751, *Acetobacter suboxydans IFO* 3131, *Vibria Metschnikovii* IAM 1039, *Actinoplanes philippinensis* (Couch, 1950, KCC ACT—0001), *Actinoplanes armeniacus* (Kalakoutskii et Kuznetsov, 1964, KCC ACT—0070), *Streptosporangium vulgars* (Nomonuracto Hara, 1960 KCC ACT—0028) or *Streptosporangium roseum* (Couch, 1955 KCC ACT—0005) under aerobic conditions, at 28 to 32°C, for at least 2 days, in a liquid medium containing a carbon source, an organic or inorganic nitrogen source, and inorganic salts, and thereafter recovering the α-1,6-glucosidase from the culture.

The microorganisms which can be used in the process of the invention can be identified by the following characteristics:—

Corynebacterium sepedonicum IFO 3306

Rods 0.3 to 0.4 by 0.8 to 1.0 micron. Pleomorphic

Non-motile. Gram-positive.

Gelatin: Liquefaction slight.

Agar colonies: Thin, smooth, translucent, glistening, whitish, 2 to 3 mm in diameter.

Broth: Weak growth. No pellicle. Light sediment.

Litmus milk: Little change in 6 weeks, after which litmus is reduced.

Indole not produced.

Hydrogen sulfide production feeble, if at all.

Glucose, galactose, fructose, arabinose, xylose, manitol, glycerol and dulcitol are utilised.

Starch hydrolysis light.

Grows in 4 per cent salt.

Temperature relations: Optimum, between 20° and 23°C. Minimum, 4°C. Maximum, 31°C.

Aeromonas hydrophila IFO 3820 (NRRL, B—909)

Rods, 0.6 by 1.3 microns, occurring singly and in chains.

Motile, with a single polar.
Gelatin colonies; Small, circular, gray translucent, stippled.
Gelatin stab: Napiform liquefaction.
Agar colonies: Whitish, raised, moist, stippled.
Horse blood agar colonies: 2 to 3 mm in diameter, round entire, raised, smooth, moist, semi-translucent, grayish white, forming a dirty brown-yellow coloration after 3 to 5 days at room temperature; marked hemolysis.
Agar slant: Thin, whitish, glassy, spreading, becoming yellowish and opalescent.
Broth: Turbid, with heavy pellicle.
Loeffler's serum: Growth abundant, but no digestion.
Litmus milk: Acid; coagulated; peptonized.
Potato: Yellowish brown, moist, slightly raised.
Indole is produced.
Nitrites produced from nitrates.
Ammonium sulfate, uric acid and asparagine may serve as sources of nitrogen.
Catalase produced.
Hydrogen sulfide produced.
Urea not attacked.
Methylene blue reduced.
Acid and gas from glucose galactose, fructose mannose, maltose, sucrose, mannitol, glycerol and starch. Acid and gas from salicin at 22°C but not at 37°C. Acid from glycogen and dextrin. Glucose fermented with the production of 2,3-butanedio. Lactose, arabinose, raffinose, rhamnose, dulcitol, sorbitol and inositol not attacked.
Starch hydrolyzed.
Gas Ratio $H_2:CO_2 = 1:4.71$. Methyl red negative, acethlmethylcarbinol positive, indole negative, citrate positive.
Produces a characteristic black-rot in hen eggs.
Aerobic, facultative.
Optimum temperature, 37°C.

Flavobacterium esteroaromaticum IFO 3751
Rods, 0.5 to 0.7 by 1.0 to 3.0 microns, occurring singly and in pairs. Gram-negative.
Gelatin colonies: Circular, raised, yellowish growth.
Agar colonies: circular yellow.
Agar slants: Whitish yellow, glistening, raised, becoming brownish yellow.
Broth: Turbid, sediment.
Litmus milk: Slightly acid.
Potato: Yellowish, glistening, spreading, good growth.
Indole not produced.
Acid from glucose, maltose, dextrins and starch.
Aerobic, facultative anaerobic.
Optimum temperature, 30°C.

Acetobacter suboxydans IFO 3130 (NRRL, B—72)
Short Rods. Occur singly or in chains. Nonmotile.
Morphologically like *Acetobacter rancens.*
Forms a very thin, hardly visible pellicle on fluid media.
Wort agar colonies: Very small, circular slightly yellow.
Minimum nutritional requirements: Pantothenic acid, nicotinic acid, p-aminobenzoic acid, valine, alanine, isoleucine, histidine, cystine, proline, mineral salts and an oxidizable substrate such as alcohol, glucose, etc.
Acid from ethanol, propanol, glycol, glucose, glycerol and sorbitol.
Optimum temperature, 30°C.

Vibrio metschnikovii IAM 1039
Curved rods, somewhat shorter and thicker than those of Vibrio comma. Long, slender chains of cells are formed in old cultures. Motile by means of a single, polar flagellum. In the animal body the cells are nearly coccoid.
Gram-negative.
Gelatin colonies: Like those of Vibrio comma.
Gelatin stab: Rapid, napiform liquefaction.
Agar slant: Yellowish, plumose, moist, glistening growth.
Broth: Turbid, with thin, white pellicle.
Litmus milk: Acid, coagulated; not peptonized.
Potato: Delicate, brownish growth.
Indole produced.
Nitrites produced from nitrates.
Aerobic facultative.
Optimum temperature, 37°C. Maximum, less than 45°C.

Actinoplanes philippinensis Couch, 1950 KCC ACT—0001 (ATCC 12427)

The mycelium on sterile Paspalum grass in water forms a very delicate, hyraline to pinkish buff internal mycelium and an inconspicuous external fringe of threads around the entire edge of the leaf; compact mounds or tufts of hyphae which are scattered over the top surface giving the leaf a speckled or finely powdery appearance are also sometimes formed. The hyphae are 0.5 to 1.5 microns wide, branched and sparingly septate. Sporangia are usually abundantly formed on grass after about ten days, usually on long unbranched stalks; they are mostly spherical when mature and measure 8.4 to 22.0 microns, most of them being about 12.0 microns in diameter on grass. At maturity the spores are arranged in coils or are irregularly placed in the sporangium; they are about 1.0 to 1.2 microns in diameter and are discharged through a pore or by the partial dissolution of the sporangial wall, swimming vigorously.

Czapek agar: Growth at room temperature poor to fair, rarely good; flat or slightly elevated; sometimes in two distinct planes; one within the agar, the other at the surface. Margin smooth or scalloped. Light buff to tawny, changing in some old cultures to Mars

brown with a lighter margin (colors as in Ridgway, Color Standards and Color Nomenclature, Washington, D.C., 1912). Sectoring frequent. In section, the growth consists of a compact surface layer, made up mostly of distinct palisades, and a submerged region of loosely arranged hyphae; the surface region is frequently stratose in old cultures with narrow, orange-colored layers. Sporangia are fairly abundant in some cultures and are not formed in others; they are spherical to irregular, frequently occurring beneath the surface in old cultures owing to overgrowth by palisade hyphae; sometimes a new layer of sporangia is formed over the first layer. Odor slightly fragrant. The agar is usually colored pale yellow.

Peptone Czapek agar: Growth good to very good, consisting of heaped convolutions in the center, becoming concentric rings of narrow ridges with narrow radial grooves, towards the outside, usually with an elevated or radially ridged-and-grooved margin. Surface shiny. Color brilliant, near apricot-orange or orange-chrome. Sporangia absent to very rare. Palisades not formed. Smaller hyphae form vast numbers of bacteroid spheres and rods which, when the material is crushed, break off and resemble Nocardia. Odor as on Czapek agar.

Potato glucose agar: Growth good to very good. Central area with coarse convolutions or large bumps and irregular ridges separated by radial grooves which slope to the smooth distinct margin. Surface glossy. Apricot-orange to russet, becoming bay in old cultures. Produces a diffusible pigment which darkens the agar. Sporangia are formed on the margin of some cultures but are absent in most of them. Palisades are formed.

Nutrient agar: Growth fair. Center slightly elevated and with a wide flat margin. Color ochraceous orange to cinnamonrufous. Sporangia very rarely formed. Palisade hyphae usually not distinct.

Krainsky's glucose asparagine agar: Growth good to very good, consisting of a central area of elevated, fine convolutions, radial ridges or bumps and a smooth area with radial grooves gradually sloping into the submerged margin. Surface moist-appearing and glossy. Color of center apricot-orange to Sayal brown surrounded by an ochraceous salmon or light ochraceous salmon margin. Sporangia are usually on the smooth areas, none being found on the elevated parts; they are formed on palisade hyphae.

Actinoplanes armeniacus KCC ACT—0070

Hyphae are 0.5 to 1.5 microns wide and branched. Branching mycelium is produced in young cultures and does not disintegrate as the cultures ages. Coiled conidiophores are lacking. Sporangiospores, 1 to 1.5 microns in diameter, are produced in coiled chaines or irregularly within sporangia; they are motile and germinate to produce the branched mycelium. Sporangia are 8.4 to 20 microns in diameter, produced on unbranched hyphae; they are predominantly spherical on long unbranched stalks.

Czapek agar medium: Growth at room temperature poor to good; flat or slightly elevated. Margin smooth or scalloped. Surface glossy; waxy to silky. Light white to pale white, changing in some old cultures to pale yellowish white. In section, the growth consists of a compact surface layer, made up mostly of distinct palisades.

Potato glucose agar: Surface glossy. Color white to yellowish white. Slightly produces dark brown diffusible pigment.

Optimum temperature, 26°C.

Streptosporangium vulgare KCC ACT—0028

Aerial mycelia 0.7 to 1.0 microns in width. Branching mycelium is produced in young cultures and does not disintegrate as the culture ages. Spherical, nonmotile condia, 1.0 to 1.2 by 1.5 to 1.9 microns, are produced in coils within sporangia. The sporangia are 6 to 8 microns in diameter and are produced apically on simple or branched aerial hyphae. Cohidia are forcibly ejected from a protuberance that forms on the sporangia when the latter are immersed in water.

Oatmeal Bacto-Yeast extract agar: Aerial mycelium pale pink to pink; soluble pigment pale yellow to yellow; no crystals of pigment produced after one month at 30°C.

Oatmeal-polypeptone agar: Substratum mycelium yellowish orange or orange after 15 days.

Starch-yeast extract agar plate: Aerial mycelium none or scanty.

Substratum mycelium yellow with orange tinge or pale orange after 15 days.

Yeast extract Glycerol-asparagine agar plate: Substratum mycelium pale rose to pale orange.

Potato agar: Growth pale yellow with orange tinge or pale orange.

Soluble pigment may be pale yellow.

Glycerin agar: Growth faire to moderate.

Nutrient agar: Growth faire to moderate.

No violet crystal produced on any medium.

Melanoides not produced.

Starch hydrolysed.

Gelatin liquefied fair or slightly.

Milk peptonized.

Nitrites not produced from nitrates.

Thiamine required essentially and biotin not required.

Streptosporangium roseum Couch, 1955 KCC ACT—0005 (ATCC 12428)

On sterile leaves, either in soil water or on damp sterile soil, a vegetable mycelium is formed which spreads over the surface of the leaf, not penetrating or decolorizing it, and

also over the soil; an aerial reproductive mycelium which is white at first but which soon changes to pale pink is also formed. The aerial mycelium appears as single hyphae or as minute tufts which grow to form mounds, up to 2mm in diameter, arranged more or less in concentric circles; the mounds usually become minutely pockmarked. Sporangia first appear on scattered single hyphae, apocal on the main thread, or on short lateral branches, a few to many sporangia on one hypha; they are formed in the hyphal tufts and mounds until the latter may be almost solid masses of sporangia. The sporangia are white in small groups, pink in large masses and spherical, measuring 7 to 19 microns in diameter on leaves, most measuring 8 to 9 microns. Shortly after their formation, spores are visible as a single coil in each sporangium; when completely formed, they are irregularly arranged. Immersion of the mature sporangium in water brings about the swelling of an intersporal substance; this swelling causes the wall and spores to push out on one side forming a cone-shaped projection about half as long as the diameter of the sporangium. The spores are forcibly ejected through an opening in the cone; they are spherical, 1.8 to 2.0 microns in diameter, possess a shiny globule and are non-motile. The sporangial wall persists for several hours after spore discharge. In addition to sporangia, conidia are formed in coils somewhat as in Streptomyces, though the coils are much less conspicuous.

Czapek agar: Growth fair, about 0.7 to 1.2 cm in diameter after 6 weeks; usually flat, level with agar surface; concentric zonation distinct or absent; central region usually compact with a broad fringed border and a tasseled edge. Surface glossy or powdery. Color usually white, sometimes pinkish buff or cream-buff. Sporangia, absent to fairly abundant, are always formed some distance above the surface of the agar. In some cultures coils are formed which break up into conidia as in Streptomyces. Palisades are absent.

Peptone Czapek agar: Growth good, about 1.5 to 2.0 cm in diameter after 6 weeks; flat or with a few low radial or irregular ridges and grooves; margin fringed or intire; aerial hyphae often formed in white concentric rings, sometimes giving a powdery appearance to the normally glossy surface. Olive-buff to deep olive-buff. Sporangia very rare.

Potato glucose agar: Growth usually good, 1.0 to 1.8 cm in diameter after 2 months; center elevated with irregular bumps and ridges; margin flat and even with surface of agar. Color at first creamy, becoming tawny and then Carob brown or Kaiser brown, after which white floccose spots of hyphae appear, usually spreading to cover the entire culture. Sporangia are usually formed in vast numbers, the white areas becoming rosy pink as the sporangia mature; the pinkish areas are frequently minutely pocked. Surface moist at first, appearing dry and floccose as aerial hyphae and sporangia are formed. Agar coloured reddish brown with a vinaceous tinge.

Agar: Growth fair, 0.7 to 1.3 cm in diameter after 2 months; central region elevated into irregular ridges which merge, towards the outside, into radial ridges and grooves sloping abruptly to the narrow, flat border; margin lobed. Usually cream-buff, rarely buffy brown. Surface usually glossy, sometimes powdery with aerial hyphae which may be united to form many upright fascicles. Sporangia is absent.

Krainsky's glucose asparagine agar: Growth poor, 0.3 to 0.7 cm in diameter; slightly elevated and minutely ridged, sloping to the fimbriate margin. Surface of central region minutely powdery with aerial hyphae. White. Sporangia absent.

Emerson's agar: Growth good, about 2 cm in diameter after 6 weeks, composed of a whitish central area, 4 to 6 mm wide, made up of elevated, irregular bumps and ridges which abruptly change into radial ridges and grooves sloping down to a flat, white border, 1 to 2 mm wide and composed of minute, concentric circles of white hyphae. Ridges and grooves vinaceous brown, sometimes covered with a whitish down. Margin smooth or scalloped, ending abruptly. Surface dry. Sporangia formed abundantly, appearing first in the center as the white changes to pink. Agar colored pale vinaceous brown.

The ability of these strains to produce α-1,6-glucosidase can be observed by carrying out the following Experiment (A).

EXPERIMENT

A. A liquid medium comprising liquefied starch as the carbon source, peptone, yeast extract, as nitrogen sources, and $MgSO_4$, KCl, K_2HPO_4, $FeSO_4$ as inorganic salts is employed. In the case of Actinoplanes and Streptosporangium a liquid medium comprising soluble starch, meat extract, peptone, and NaCl can be used. 5 ml of the medium is transferred into test tubes (18 x 180 mm). After a loopful of inoculum grown in agar slant medium has been inoculated on the medium, the medium was cultivated at 30°C for 4 days under shaking conditions.

Pullulanase activity can be assayed by the following method. The reaction mixture used consists of 0.5 ml of culture broth or culture filtrate obtained as described above, 0.5 ml of acetate buffer (pH 5.2), and 1.0 ml of 1% pullulan solution. After incubation at 40°C for 1 hr., 4.0 ml of absolute alcohol is added to discontinue the reaction. The precipitate is filtered on filter paper after standing at a low temperature overnight. The reducing sugars produced in the filtrate are determined by the Anthrone method. Then enzyme blank (0.5 ml of enzyme solution+0.5 ml of N/2

Acetate buffer (pH 5.2)+1.0 ml of water) and substrate blank (1.0 ml of 1% pullalan+0.5 ml of N/2 Acetate buffer (pH 5.2)+0.5 ml of water) are incubated by the same method. With the addition of 4 ml of absolute alcohol the reaction is suspended. The reaction mixture is treated by the above method and the amount of sugars present are determined. One unit of isoamylase activity per millilitre of enzyme solution was defined as the amount of enzyme in the reaction mixture required to produce a difference of 180 μ g (as glucose) /ml (reaction mixture) between the amounts of reducing sugars in the reaction mixture after incubation at 40°C for one hour and the amount of reducing sugars in the enzyme blank plus substrate blank after similar incubation.

The results are given in Table 1. [O.D.] × 1/10 represents the optical density at 660 mμ with a photocell, 1 cm wide, of 10 fold-diluted culture broth. The units/ml determined by the above method are equal to the units per millilitre of isoamylase activity detemined by a modified version of the method of Maruo and Kobayashi (Bull. Agr. Chem. Soc. Japan 19, 163—166, 1955). One unit of isoamylase activity determined by this modified method is equal to about 2.5 units of that determined by the method of Maruo and Kobayashi.

TABLE 1

Type culture	pH	O.D. × 1/10	units/ml
Corynebacterium sepedonicum IFO 3306	8.7	0.376	2.7
Aeromonas hydrophila	8.6	0.420	9.8
Flavobacterium esteroaromaticum IFO 3751	5.8	0.780	3.5
Acetobacter suboxydans IFO 3130	3.6	0.380	1.3
Vibrio metschnikovii IAM 1039	6.1	0.254	5.4
Actinoplanes phillippinensis Couch, 1950, KCC ACT—0001	5.6	0.450	13.0
Actinoplanes armeniacus Kalakoutskii et Kuznetsov, 1964, KCC ACT—0070	6.2	0.370	4.0
Streptosporangium vulgare Nomonuraeto Hara, 1960 KCC ACT —0028	6.0	0.341	1.0
Streptosporangium roseum Couch, 1955 KCC ACT—0005	5.8	0.410	6.1

B) *Preparation of enzyme solutions*

Culture was performed with the employment of strains listed in Table 1. The liquid medium, which was used, comprised maltose, soluble starch, liquefied starch as carbon sources, peptone, yeast extract and CH_3COONH_4 as organic nitrogen sources, $NaNO_3$, $(NH_4)_2SO_4$, as inorganic nitrogen sources, and $MgSO_4$, KCl, K_2HPO_4, $FeSO_4$. The mixture was cultivated in an Erlonmeyer flask at 28—32°C for 2—4 days under rotary shaking conditions.

In the case of the microorganisms of the genera *Actinoplanes* and *Streptosporangium* soluble starch, liquefied starch were used as carbon sources and peptone, yeast extract, meat extract and ammonium salts were used as nitrogen sources together with NaCl and other inorganic salts. Inoculum from fresh slant cultures was inoculated on the liquid medium, which was cultivated at 30°C for 4—10 days under shaking conditions.

The above before mentioned method for determining the activity of the enzyme using pullulan, as a substrate was preferable.

Since the enzyme produced is cell free or cellbound enzyme, the enzyme activity may be

determined from, and utilised in the form of the culture filtrate or supernatant solution obtained by centrifugation, or as the enzyme extract obtained by treatment of the culture broth or harvested cells by an ultrasonic oscillator or autolyzates such as Sodium-lauryl-sulphate (S.D.E.) and toluene. The culture broth was separated into cell and supernatant solution by centrifugation. The cellbound enzyme was obtained by suspending the cells in 0.1% SDS—Mac Ilvain Buffer solution (pH 7.0) and autolysating at 30°C for 2 days under shaking conditions. The cell-free enzyme was obtained by salting out by adding ammonium sulphate to 90% saturation, and collecting the precipitate. The activity of the cell-bound or cell-free enzyme solution was measured. The results are shown in Table 2.

TABLE 2

Type culture	Pullulanase Activity (a shake culture, 30°C, 4 days)		
	Cell-bound enzyme activity	Cell-free enzyme activity	Total activity
Corynebacterium sepedonicum IFO 3006	2.2 units/ml	0.0 units/ml	2.2 units/ml
Aeromonas hydrophila IFO 3820	4.5 „	8.7 „	13.2 „
Flavobacterium esteroaromaticum IFO 3751	6.1 „	0.0 „	6.1 „
Acetobacter Suboxydans IFO 3130	1.6 „	0.0 „	1.6 „
Vibrio metschnikovii IAM 1039	5.0 „	0.0 „	5.0 „

The results obtained with *Actinomycetes* after incubation at 30°C for 4 — 10 days are listed in Table 3.

TABLE 3

Type Culture	Culture period	Pullulanase Activity units/ml		
		Cell-free enzyme activity	Cell-bound enzyme activity	Total activity
Actinoplanes philippinensis KCC ACT—0001	4 days	6.5	7.5	13.8
	10 days	25.0	—	25.0
Streptosporangium roseum KCC ACT—0005	4 days	1.5	—	1.5
	10 days	2.0	—	2.0

Since a slight α-amylase activity was recognised in the above enzyme solutions, the α-amylase activities were determined.

To a mixture comprising 5 ml of 1% soluble starch and 4 ml of M/8 acetate buffer (pH 6.0) was added 1.0 ml of enzyme solution diluted to provide an α-amylase activity of 0 to 0.2 units (Lintner Solied Value) per millilitre. After incubating the solution for certain periods at 40°C, 0.5 ml of the solution was withdrawn and mixed with a solution comprising 15 ml of N/100 H_2SO_4 and 0.5 ml of M/100 I_2—KI solution. After standing for 15 minutes at room temperature the decrease in optical density at 610 mμ with the use of a 1 cm cell was measured. When the decrease in optical density after 10 minutes of incubation was 0.110 from the start of incubation, the α-amylase activity was equal to about 0.1 units per millilitre of Lintner Soleid value. When the activity was determined under conditions in which the α-amylase has a concentration of 0—0.2 units (LSV)/ml and which produce a decrease in optical density lower than 0.450, the decrease in optical density was proportional to the concentration of enzyme and to the reaction time. Thus reactions were performed under these conditions. The values obtained by determination of α-amylase activity included the increase in optical density which resulted from the action of α-1,6-glucosidase on the substrate. However, for practical purposes such increases of optical density due to the α-1,6-glucosidase were negligible and the values obtained were allowed to remain uncorrected. Typical results for α-amylase activity are shown in Table 4.

TABLE 4

Type culture	α-Amylase activity [units(LSV)/ml]			
	Cell-free enzyme activity (units/ml)	Cell-bound enzyme activity (units/ml)	Salting out-enzyme activity (units/ml)	SDS-extracts (units/ml)
Corynebacterium spedonicum IFO 3306	0.16 (600 ml)	—	1.26 (76 ml)	—
Aeromonas hydrophila IFO 3820	—	0.016 (710 ml)	—	0.23 (50 ml)
Flavobacterium esteroaromaticum IFO 3751	0.000	—	0.007 (20 ml)	—
Acetobactrr suboxydans IFO 3130	0.051	—	2.29	—
Vibrio metschnikovii IAM 1039	—	0.001	—	0.014

C) *Actions on various substrates*

(a) A reaction solution comprising 0.5 ml culture broth obtained as described in the above Experiment A, 1.0 ml of 1% pullulan solution, 0.5 ml of N/2 acetate buffer (pH 5.2) was incubated in a test tube at 40°C for 16 hours and then thin-layer chromatographed. *Aeromonas hydrophila* IFO 3820 and *Actinomycetes* displayed distinctive spots mainly of maltotriose on the chromatograms.

Other strains were found to produce maltotriose and to hydrolyse the α-1,6-glucosidic linkages of pullulan.

(b) As for the action of *Aeromonas hydrophila* IFO 3820 on amylopectin, 0.5 ml of the enzyme solution purified by ion exchange absorbents was incubated with a mixture solution of 1.0 ml of 1% amylopectin solution and 0.5 ml of N/2 acetate buffer (pH 5.2) at 40°C for 16 hours. The reaction mixture produced a deep blue

colour when stained with N/10 I_2—KI solution and showed formation of amylodextrin as a result of hydrolysis of the α-1,6-linkages. The absence of α-1,6-linkages was confirmed by the formation of 95—98% maltose by treating the resultant hydrolysate with β-amylase.

D) *Properties of the enzymes*

In the prepared enzyme solution other amylases which hydrolyze starch are present in a mixed state. However, since the other amylases have no effect on pullulan, the optimum pH and optimum temperature of α-1,6-glucosidase in the prepared enzyme may be measured using salting out enzyme solutions on pullulan. Determination was performed using the above mentioned reaction solution constituents and varying the pH, and temperature, or occasionally varying the reaction time. The activities and kinds of enzyme solutions are shown in Table 5.

TABLE 5

Type culture	Varieties of enzymes used and their activities	Reaction time
Corynebacterium sepedonicum IFO 3306	Diluted salting out-enzyme solution (3 units/ml)	3 hrs.
Aeromonas hydrophila IFO 3820	Diluted SDS-extract solution (25 units/ml) Diluted salting out-enzyme solution (4.8 units/ml)	1 hr.
Flavobacterium esteroaromaticum IFO 3751	Diluted salting out-enzyme solution (4.0 units/ml)	3 hrs.
Acetobacter suboxydans IFO 3130	Diluted salting out-enzyme solution (2.5 units/ml)	1 hr.
Vibrio metschnikovii IAM 1039	Diluted salting out-enzyme solution (3.0 units/ml)	1 hr.
Actinoplanes philippinensis Couch, 1950 KCC ACT-0001	Diluted salting out-enzyme solution (3.38 units/ml)	1 hr.
Streptosporangium roseum Couch, 1955 KCC ACT-0005	Diluted salting out-enzyme solution (1.17 units/ml)	16 hrs.

Determination of the optimum pH was performed at 40°C with pHs of 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 and that for the optimum temperature at pH 5.2 with temperatures of 15, 35, 40, 45, 50, 55 and 60°C. The reaction time is given in Table 5.

The results for determination of the optimum pH and optimum temperature are summarised in Table 6.

TABLE 6

Type culture	Opt. pH	Opt. Temp.
Corynebacterium sepedonicum IFO 3306	6.0	50 – .55°C
Aeromonas hydrophila IFO 3820	5.0	55°C
Flavobacterium esteroaromaticum IFO 3751	8.0	35°C
Acetobacter suboxydans IFO 3130	6.0	40°C
Vibrio metschnikovii IAM 1039	5.5	45°C
Actinoplanes philippinensis KCC ACT-0001	7.0	45°C
Streptosporangium roseum KCC ACT-0005	6.0	45°C
cf. *Aerobacter aerogenes* (Wallenfels & Bendar 1961)	5.0	47.5°C

EXAMPLE 1.

Strains used: *Corynebacterium sepedonicum* IFO 3306, *Aeromonas hydrophila* IFO 3820, *Flavobacterium esteroaromaticum* IFO 3751, *Acetobacter suboxydans* IFO 3130, *Vibrio metschnikovii* IAM 1039.

Culture: On 100 ml of medium of the following composition as in Experiment A, i.e. 1% liquefied starch, 1% peptone, 0.5% yeast extract, 0.1% K_2HPO_4, 0.05% KCl, 0.01% $MgSO_4 . 7H_2O$, 0.001% $FeSO_4 . 7H_2O$ (pH 7.0), strains were inoculated from fresh slant culture and incubated at 30°C for 4 days under shaking conditions. The activities after incubation are given in Table 7.

Determination of Pullulanase activity: Performed exactly as described in Experiment A.

Preparation of enzyme solutions: Cells harvested by centrifuging the culture broth were suspended with 0.1% SDS—Mac Ilvain buffer solution (pH 7.0). The autolyzate obtained by shaking the suspesnion for 2 days at 30°C was used as enzyme solution and its activity was determined. The supernatant solution obtained by centrifuging the culture broth was salted out against by adding ammonium sulphate to 90% saturation. Precipitates which were collected and suspended in pure water were used as enzyme solution and its activity was determined. Examples of enzyme preparations of various strains are shown in Table 7.

TABLE 7

Type culture	Supernatant of broth		Cell-SDS-extract soln.		Salting out-enzyme soln.	
	Vol. (ml)	Pullulanase activity (units/ml)	Vol. (ml)	Pullulanase activity (units/ml)	Vol. (ml)	Pullulanase activity (units/ml)
Corynebacterium sepedonicum IFO 3306	600	1.2	50	0.00	20	35
Aeromonas hydrophila IFO 3820	710	4.4	50	12.40	200	15.0
Flavobacterium esteroaromaticum IFO 3751	900	6.1	50	0.00	200	26.0
Acetobacter suboxydans IFO 3130	900	0.5	50	0.00	20	21.0
Vibrio Netschnikovii IAM 1039	890	4.1	50	0.00	50	70.0

EXAMPLE 2.

The strains used in this Example were the following:

Actinoplanes philippinensis Couch, 1950 KCC ACT—0001
[←Couch P—15 (ATCC 12427) (USSR RIA 4687)]

Streptosporangium roseum Couch, 1955 KCC ACT—0005
[←Couch 27 b (ATCC 12428) (IFO 3776) (USSR RIA 470)]

Culture was performed by inoculating strains from fresh slant culture on 100 ml liquid medium in a 500 ml flask which comprises, 2% soluble starch (liquefied to violet I_2—KI stain), 0.5% meat extract, 0.5% peptone, and 0.5% NaCl, (pH 7.4) and cultivating under shaking conditions.

Preparation of enzyme solutions: One tenth of the above culture filtrate (or supernatant solution from centrifugation of the culture) was salted out against by adding ammonium sulphate to 90% saturation. The precipitate was suspended in pure water and the suspension was used as enzyme solution and its activity was determined. In Table 8 examples of enzyme preparations of the two strains are shown. (The method of extracting cell-bound enzyme and preparation of enzyme solutions were as described in Example 1).

TABLE 8

Type culture	Supernatant of broth		Cell-SDS-extract soln.		Salting out-enzyme soln.	
	Vol (ml)	Pullulanase activity (units/ml)	Vol. (ml)	Pullulanase activity (units/ml)	Vol. (ml)	Pullulanase activity (units/ml)
Actinoplanes philippinensis Couch, 1950 KCC ACT-0001	600	12.5	66 (Data of 4 days culture)	325	200	35.90
Streptosporangium roseum Couch, 1955 KCC ACT-0005	900	1.5	— (Data of 4 days culture)	—	20	50.5

The terms "IFO", "IAM", and "KCC ACT" are respectively abbreviations for the following:—

IFO: Institute for Fermentation, Osaka, Japan.

IAM: Institute of Applied Microbiology, University of Tokyo, Japan.

KCC ACT: the deposited strain member at the Kaken Chemical Company Ltd., Tokyo 6—42, Jujodai, 1-chome, Kita-ku, Tokyo, Japan.

WHAT WE CLAIM IS:—

1. A process for the preparation of α-1,6-glucosidase comprising cultivating *Corynebacterium sepedonicum* IFO 3306, *Aeromonas hydrophila* IFO 3820, *Flavobacterium esteroaromaticum* IFO 3751, *Acetobacter suboxydans* IFO 3130, *Vibrio Metschnikovii* IAM 1039, *Actinoplanes philippinensis* (Couch, 1950, KCC ACT—0001), *Actinoplanes armeniacus* (Kalakoutskii et Kuznetsov, 1964, KCC ACT—0070), *Streptosporangium vulgars* (Nomonuracto Hara, 1960 KCC ACT—0028) or *Streptosporangium roseum* (Couch, 1955 KCC ACT—0005) under aerobic conditions, at 28 to 32°C for at least 2 days, in a liquid medium containing a carbon source, an organic or inorganic nitrogen source, and inorganic salts, and thereafter recovering the α-1,6-glucosidase from the culture.

2. A process for the preparation of α-1,6-glucosidases according to Claim 1, substantially as herein described.

3. A process for the preparation of α-1,6-glucosidases according to Claim 1, substantially as herein described, with reference to the Examples.

4. α-1,6-glucosidases whenever prepared by a process according to any one of Claims 1 to 3.

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